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BLOOD SCHIZONTOCIDAL ACTIVITY OF METHYLENE BLUE IN COMBINATION WITH ANTIMALARIALS AGAINST *PLASMODIUM FALCIPARUM*

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Summary:

Methylene blue (MB) is the oldest synthetic antimalarial. It is not used anymore as antimalarial but should be reconsidered. For this purpose we have measured its impact on both chloroquine sensitive and resistant *Plasmodium* strains. We showed that around 5 nM of MB were able to inhibit 50 % of the parasite growth *in vitro* and that late rings and early trophozoites were the most sensitive stages; while early rings, late trophozoites and schizonts were less sensitive. Drug interaction study following fractional inhibitory concentrations (FIC) method showed antagonism with amodiaquine, atovaquone, doxycycline, pyrimethamine; additivity with artemether, chloroquine, mefloquine, primaquine and synergy with quinine. These results confirmed the interest of MB that could be integrated in a new low cost antimalarial combination therapy.

KEY WORDS : antimalarials, *Plasmodium*, methylene blue, drug interactions.

Résumé : ACTIVITÉ DU BLEU DE MÉTHYLENE EN COMBINAISON AVEC DES ANTIPALUDIQUES CONTRE LES STADES SANGUINS DE *PLASMODIUM FALCIPARUM*

Le bleu de méthylène (BM) est l'antipaludique de synthèse le plus ancien. Il n'est désormais plus employé comme antipaludique, mais son usage devrait être reconsidéré. Nous avons mesuré son effet sur des souches de *Plasmodium* chloroquino-sensibles et chloroquino-résistantes. Nous avons montré que 5 nM de BM inhibent 50 % du développement parasitaire *in vitro* et que les stades anneaux mûrs et trophozoites jeunes sont les stades les plus sensibles tandis que les trophozoites âgées, les schizontes et les anneaux jeunes sont les moins sensibles. L'étude de l'interaction de drogues selon la méthode des concentrations inhibitrices fractionnelles ou FIC montre que l'association avec le MB est: antagoniste avec l'amodiaquine, l'atovaquone, la doxycycline et la pyriméthamine, additive avec l'artéméthér, la chloroquine, la méfloquine et la primaquine et synergique avec la quinine. Ces résultats confirment l'intérêt du BM qui pourrait être intégré dans une nouvelle thérapie antipaludique de combinaison à prix réduit.

MOTS CLÉS : antipaludiques, *Plasmodium*, bleu de méthylène, interaction de drogues.

INTRODUCTION

Malaria remains one of the most important parasitosis in tropical developing countries, killing 1-3 million people (mostly children) in sub-Saharan Africa, and causing disease in 300-500 million people a year. Despite international commitment to control and research, the reality for many patients is much as it was twenty years ago: they still die because being unable to access treatment for economic reasons. Moreover available drugs are not efficient due to parasite resistance (Olliaro, 2005). In this context, new, safe, affordable and efficient drugs against resistant strains of *Plasmodium* are needed. In addition to be effective against drug resistant malaria, an ideal new can-

didate must be effective within a three-day regimen, safe in small children (< 6 months) and pregnant women, easy to package, cheap and have a low propensity to generate resistance (Baird, 2005; Pink *et al.*, 2005).

Adapted to oral administration, MB gathers all these features except one: it is not new anymore. In 1891, Ehrlich & Guttman (1891) used MB to treat two patients infected by *Plasmodium*; for the first time a synthetic drug was shown to be active against malaria. Later, MB along with quinine, served as structural starting points for the development of 8-aminoquinoline (pamaquine) and 9-aminoacridine (mepacrine, quina-crine) in 1925 and 1930, respectively (Schulemann, 1932). Since that time, few works on the antimalarial properties of MB have been reported because it was thought to enhance hemolysis in case of glucose-6-phosphate-dehydrogenase (G6PD) deficiency (Schirmer *et al.*, 2003) and could stain the tissues of MB-treated people (Wainwright & Amaral, 2005). Nevertheless, Mandi *et al.* (2005) and Meissner *et al.* (2005) found recently that G6PD deficiency did not compromise the use of MB for malaria treatment, even in class III G6PD deficient young children. Recently, Akoachere *et al.* (2005) studied the interaction between MB and clas-

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sical antimalarial drugs, and found MB to be antagonistic with amodiaquine and chloroquine. This sounded strange to us, as MB is an exceptionally weak base (pKa 0-1) (Keene *et al.*, 1965; Pottier *et al.*, 1975) accumulating inside malaria-infected red blood cell in which it is reduced and probably concentrated in the food vacuole where it inhibits the formation of hemozoin (Atamna *et al.*, 1996), just as 4-aminoquinolines do. Moreover, MB inhibits parasite glutathione reductase thus jeopardizes glutathione functionality (Färber *et al.*, 1998). Instead of antagonise, this phenomenon should enhance chloroquine activity, which is closely related to glutathione levels (Meierjohann *et al.*, 2002). We thus re-examined herein the activity of MB on African and South-American *P. falciparum* strains, determined the most sensitive parasitic stage and studied the association of MB with classical antimalarial drugs, as the spread of multidrug-resistant *Plasmodium* strains favours the use of combinations to protect the individual compounds from emergence of resistance [WHO-TDR, 2006].

MATERIALS AND METHODS

CHEMICALS

All chemicals were from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France) except artemether (ART) from Cambrex (Verviers, Belgium), atovaquone (ATO) was a gift from GlaxoSmithKline (Marly-le-Roi, France) and mefloquine (MEF) from Hoffmann-La Roche (Basel, Switzerland).

Amodiaquine (AMO), ART, ATO, chloroquine (CQ), doxycycline (DOX), MB, primaquine (PRIM), pyrimethamine (PYR), quinine (Q) and MEF were dissolved in appropriate solvents and then with RPMI 1640 medium (Cambrex). All final concentrations of solvent were < 0.01 %, as recommended by Ye *et al.* (1983).

PLASMODIUM FALCIPARUM IN VITRO

Four uncloned strains of *P. falciparum* were used: F32-Tanzania, HB3-Honduras, FcM29-Cameroon and FcB1-Colombia. Parasites were cultured according to Trager and Jensen (1976) on human type O+ erythrocytes in RPMI 1640 medium supplemented with 25 mM HEPES buffer, 10 % AB human serum (3-5 % hematocrit), 2 mg of sodium bicarbonate/ml, 0.5 µg of gentamicin/ml and incubated at 37°C in a reduced oxygen environment (e.g. a custom mixture of 5 % CO₂, 5 % O₂ and 90 % N₂).

BIOASSAYS

The *in vitro* drug study was adapted from the microdilution technique of Desjardins *et al.* (1979). Each drug was tested for 48 h in triplicate in 96-well plates (TPP,

Switzerland) with culture at ring stage (synchronization by 5 % D-sorbitol lysis; Sigma-Aldrich) and 0.5-2 % parasitemia (hematocrit: 1.5 %). Parasite growth was estimated by [³H]-hypoxanthine (PerkinElmer, Courtaboeuf, France) incorporation (1.44 µCi/ml); plates were frozen-defrosted and each well was harvested onto a glass fibber filter (PerkinElmer). Incorporated [³H]-hypoxanthine was then determined with a β-counter (1450-Microbeta Trilux, Wallac-PerkinElmer); controls were performed to assess the background (negative control) and the parasite growth (positive control). The IC₅₀ of the different strains of *P. falciparum* were determined with a linear least square regression analysis. Stage specificity tests were performed on FcB1 strain. Drugs were tested in triplicate against a culture synchronized for a 6 h period by treatment with Plasmion (Laboratoire Fresenius Kabi, France) and consecutive 5 % D-sorbitol lysis (Lelièvre *et al.*, 2005). After each exposition, treated wells were washed twice with RPMI and cells returned to normal culture conditions until time zero plus 48 h (Valentin *et al.*, 1997). [³H]-hypoxanthine (0.5 µCi/well) was then added and plates were incubated for another 36 h. Results were expressed as percentage of inhibition *versus* controls without drug. Combination experiments were performed with FcM29 strain. Drugs were combined in 96 wells microtitration test plates at various fractions of their respective IC₅₀. Results were expressed as the mean sums of the fractional inhibitory concentrations (FIC), defined as (IC₅₀ of drug A in mixture/IC₅₀ of drug A alone) + (IC₅₀ of drug B in mixture/IC₅₀ of drug B alone) for each fixed concentration (Berenbaum, 1978; Canfield *et al.*, 1995). Three types of drug interaction were defined as follows: additivity, sum of FIC = 1; synergism, sum of FIC < 1; and antagonism, sum of FIC > 1 (Ringwald *et al.*, 1999; Ankoachere *et al.* 2005). We followed this classification to get comparable results with Ankoachere study, although Odds (2003) suggested much more restrictive definitions: synergism (FIC ≤ 0.5), no interaction (FIC > 0.5-4) and antagonism (FIC > 4) (Odds, 2003).

RESULTS

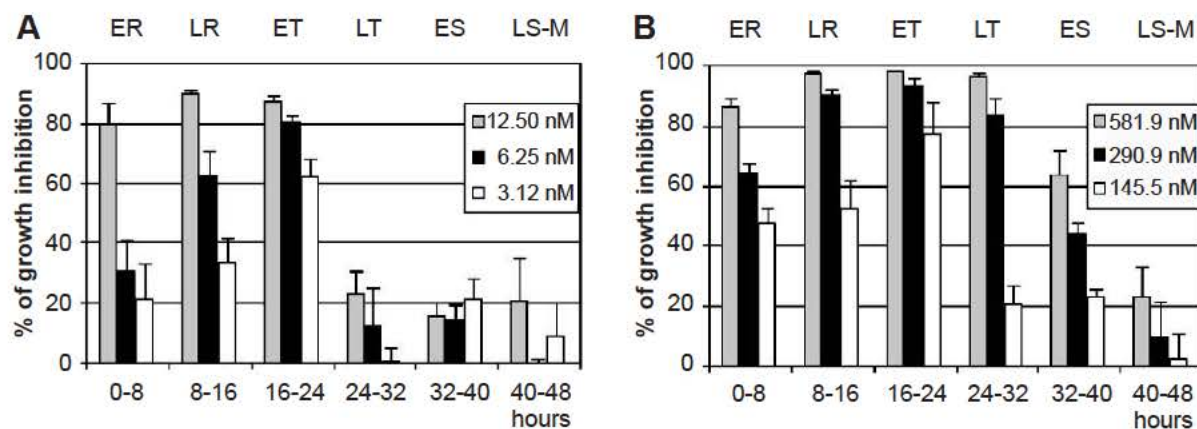
Activities of the ten drugs against the four strains of *P. falciparum* are listed in Table I. FcB1 strain was sensitive to AMO, MEF, PYR and Q and resistant to ART, ATO, CQ and DOX. The F32 strain was sensitive to AMO, ART and CQ and resistant to ATO, DOX, MEF, PYR and Q. The HB3 strain was sensitive to all the tested drugs. F32 and HB3 were similarly sensitive to CQ while FcB1 and FcM29 were resistant, FcM29 being almost three times more resistant than FcB1. FcM29 was thus selected for the drug interaction tests. It was sensitive to AMO, Q and resis-

Compounds	IC ₅₀ (nM)				Resistance threshold ^a	FIC Mean ^b FcM29	Interactions
	F32	HB3	FcB1	FcM29			
Amodiaquine	19	23	22	33	> 60	1.28 ± 0.11	Antagonism
Artemether	0,4	10	14	16	> 12	0.95 ± 0.05	Additivity
Atovaquone	20	0,1	22	188	> 7	1.24 ± 0.17	Antagonism
Chloroquine	70	36	186	600	> 100	1.03 ± 0.08	Additivity
Doxycycline	26,185	4,446	12,540	22,632	> 9,600	1.44 ± 0.28	Antagonism
Mefloquine	401	31	16	106	> 30	0.97 ± 0.12	Additivity
Methylene blue	5	4	8	5	ND	–	–
Primaquine	4,627	ND	432	13,20	ND	0.93 ± 0.08	Additivity
Pyrimethamine	6,882	ND	97	21,774	> 2,000	1.15 ± 0.10	Antagonism
Quinine	835	208	423	331	> 500	0.74 ± 0.11	Synergy

Interactions : additivity, sum of FIC = 1; synergism, sum of FIC < 1; and antagonism, sum of FIC > 1 (according to Ankoachere *et al.*, 2005).

^aInformation provided by the Centre national de référence sur la chimiorésistance du paludisme, Institut Pasteur de Guyane; ^bFIC mean = mean sum of fractional inhibitory concentrations ± SD (obtained from 5 to 20 determinations); ND: not determined.

Table I. – *In vitro* activity of MB blue and classical antimalarials (alone or in combination) against different strains of *P. falciparum*.



ER = early rings; LR = late rings; ET = early trophozoites; LT = late trophozoites; ES = early schizonts; LS = late schizonts; M = merozoites; Average ± SD.

Fig. 1. – Sensitivity of the different stages of *P. falciparum* FcB1 strain to increasing doses of methylene blue and quinine: (A) methylene blue; (B) quinine.

tant to the other drugs (except MB). MB was highly active against the four strains of *P. falciparum*, being the most active of all tested drugs. When tested on highly synchronized parasites, it appeared that late rings and early trophozoites were the most sensitive stages to MB (Fig. 1). This was similar for Q who additionally displayed good activity on late trophozoites (Fig. 1). Early rings were moderately sensitive, whereas early and late schizonts showed the lowest susceptibility for the two drugs. Late trophozoites were sensitive to Q but not to MB.

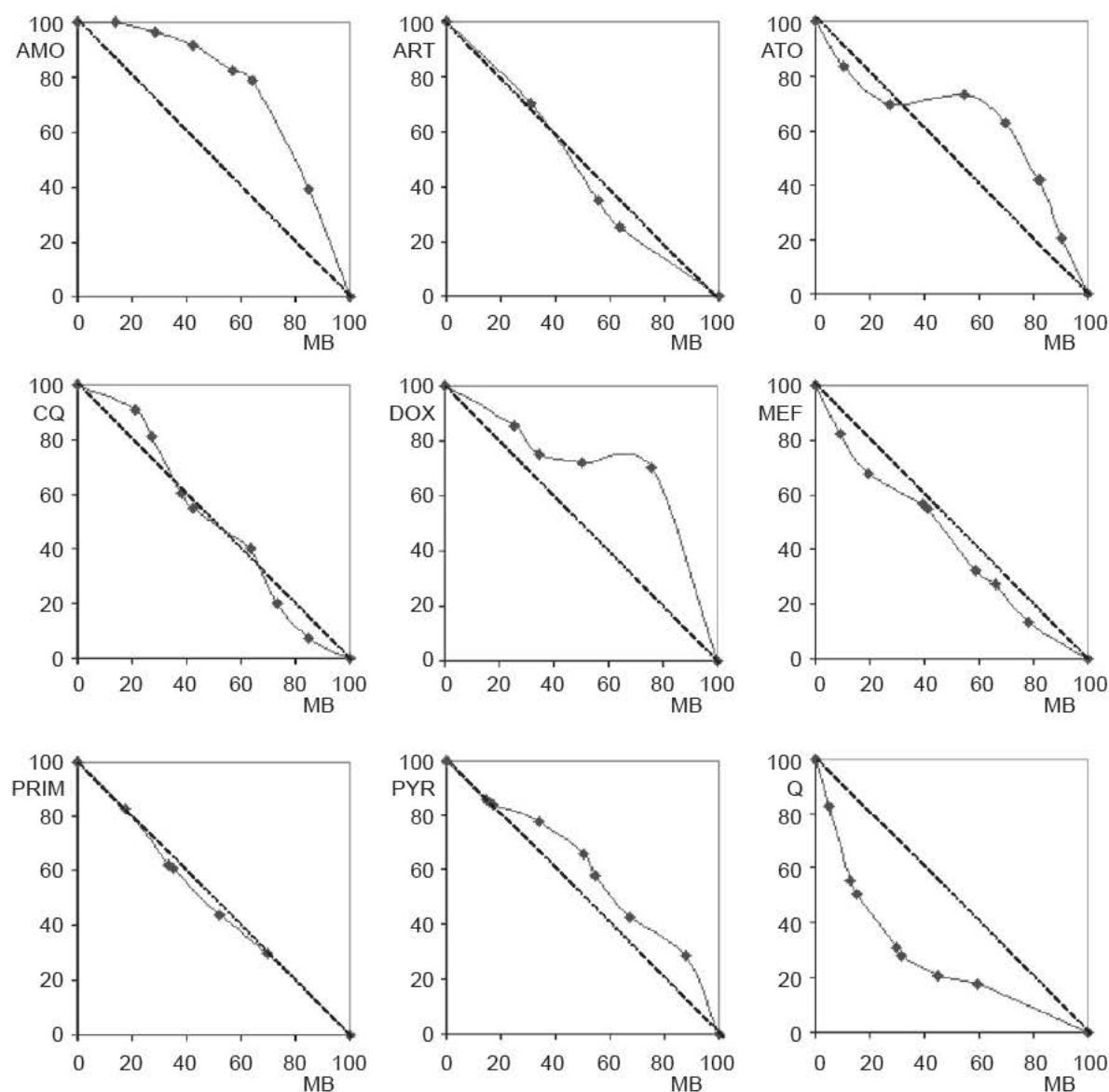
Results of the combination studies following the criteria defined by Ankoachere *et al.* (2005) indicated that interactions with MB ranged from antagonism to synergism (see Table I and Fig. 2): antagonism with AMO, ATO, DOX and PYR; additivity with ART, CQ, MEF and PRIM; and synergy with quinine. According to the scale defined by Odds (2003), there is no interaction between MB and all tested drugs.

DISCUSSION AND CONCLUSION

Methylene blue has already been shown to be active alone *in vitro* against laboratory-adapted strains from various geographic regions, with IC₅₀ ranging from 3 to 11 nM (Atamna *et al.*, 1996; Venerstrom *et al.*, 1995). Our results further demonstrated the *in vitro* activity of MB against F32 and HB3 chloroquine-sensitive strains, and FcM29 and FcB1 chloroquine-resistant strains of *P. falciparum* with the same range of activity (~ 5 nM). Akoachere *et al.* (2005) showed that MB was active against K1-South East Asia and Dd2-Indochina chloroquine-resistant strains at around 7 nM. That means that MB is strongly active against strains from all over the world including chloroquine-resistant strains. Subjecting a synchronized culture to pulses of MB every 8 h for 48 h (Fig. 1) we showed that MB inhibited around 60 % of the parasite growth between the 8th to the 24th h of the parasite cycle

(at 6.25 nM) when the production of plasmodial RNA and protein are maximal (Arnot & Gull, 1998). That is consistent with the fact that the antimalarial activity of MB depends on the *Plasmodium* antioxidant defences, which are fully functional at the late ring-early trophozoite stages when intensive digestion of haemoglobin occurs. This is also correlated with the peak of expression of plasmodial glutathione reductase (PF140192), which is targeted by MB (Färber *et al.*, 1998), starting 8 hours post invasion and reaching its maximum at the 24th hour of the intraerythrocytic cycle (PlasmoDB, 2005). With the emergence and spread of drug resistance in malaria endemic regions, combination therapy repre-

sents an effective approach in curbing the development of resistance of the parasite to the commercially available compounds (Gupta *et al.*, 2002). Additionally it may reduce toxicity and shorter treatment regimens (Pink *et al.*, 2005). Akoachere *et al.* (2005) found MB to be antagonistic with AMO, CQ, PRIM and PYR; additive with MEF and Q; synergistic with ART, artemisinin and artesunate, against *P. falciparum* K1 strain in culture. Antagonism with AMO and CQ is surprising as MB (like 4-aminoquinolines) concentrated in *Plasmodium* food vacuole where it inhibits the formation of hemozoin (Atamna *et al.*, 1996). It is also known to inhibit parasite glutathione reductase (Färber *et al.*,



The straight line in each panel represents additivity. Points located below this line indicate synergism, data points above the line indicate antagonism.

AMO = amodiaquine; ART = artemether; ATO = atovaquone; CQ = chloroquine; DOX = doxycycline; MEF = mefloquine; MB = methylene blue; PRIM = primaquine; PYR = pyrimethamine; Q = quinine.

Fig. 2. – Isobolograms of drug interactions between MB and other antimalarial drugs.

1998) enhancing chloroquine activity (Meierjohann *et al.*, 2002). We thus decided to study the interaction between MB and known antimalarials with a slightly different model from that of Akoachere *et al.* (2005). We started with higher (1-2 %) parasitemia and initiated radio labelling at time zero plus 32 h for 16 h. We ended the experiment after 48 h incubation. We found antagonism with MB + AMO, ATO, DOX and PYR while in the Akoachere's study AMO, CQ, PRIM and PYR were antagonist. Although AMO belongs to the same chemical class of compounds as CQ, the 4-aminoquinolines, the association of MB and this drug was antagonist in both studies, this must be due to the fact that the antimalarial activity of AMO is mainly exerted through its metabolite, desethylamodiaquine (Mariga *et al.*, 2005). DOX and PYR are very cheap, unfortunately these products were antagonist with MB against *P. falciparum* FcM29 strain (DOX was not tested in Akoachere's study and PYR was also found antagonist). In our hands, the association of MB with Q was synergic (additive in Akoachere's study). Q present similarities with MB: we showed that the activity of the two drugs was directed against initial stages of the blood cycle (Q being also slightly active against late trophozoites). As MB, Q is a weak membrane-soluble base, accumulating in the acidic digestive vacuole contents where it undergoes protonation. Unfortunately Q use is limited by its long therapy duration, entailing poor adherence with high risk of failure, and its potential toxicity in people with G6PD deficiency (Baird, 2005). Additive interactions were also observed between MB and ART, CQ, MEF and PRIM. ART is a drug of choice and was found to be synergic in Akoachere's study (Akoachere *et al.*, 2005). Despite a prohibitive price making their deployment difficult, artemisinin-based combination therapies (ACTs) are the best antimalarial treatments available nowadays and are recommended by WHO since 2001 (Mutabingwa, 2005). MEF was additive in both studies but it is not an optimal candidate because persistence of subinhibitory concentrations for long periods (elimination $t_{1/2}$, 2 weeks) (Karbwang *et al.*, 1988; Mansor *et al.*, 1989). CQ remains one of the cheapest antimalarial easily accessible in developing countries. Rengelshausen *et al.* (2004) showed that oral co-administration of MB and CQ did not show major pharmacokinetic interactions confirming the feasibility of this combination. Nevertheless, in a trial conducted in Burkina Faso, where high resistance of *Plasmodium* to CQ was reported, Meissner *et al.* (2006) found that the CQ-MB combination was not sufficiently effective in the treatment of uncomplicated malaria in young children. This observation was correlated with antagonism reported by Akoachere *et al.* (2005), between CQ and MB.

In conclusion, according to Akoachere *et al.* (2005) criteria: additivity with MEF and antagonism with AMO were supported in both studies. Q was synergic in our

hands and additive in their study. ART was synergic in Akoachere's study and additive for us. On the contrary we found additivity with CQ and PRIM while antagonism was claimed by Akoachere *et al.* (2005). We also showed antagonism with DOX and ATO, not tested elsewhere. These discrepancies suggest that *in vivo* studies with animal models should be conducted to determine adequate associations.

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